Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 327 (2004) 262–272

VIROLOGY

www.elsevier.com/locate/yviro

Characterization of a DNA vaccine expressing a human immunodeficiency virus-like particle

Kelly R. Young[a], James M. Smith[b], Ted M. Ross[a,*]

[a]*Division of Infectious Diseases, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[b]*Antibody Systems, Inc., Hurst, TX 76054, USA*

Received 8 April 2004; returned to author for revision 14 June 2004; accepted 12 July 2004

Abstract

An ideal human immunodeficiency virus type-1 (HIV-1) vaccine will most likely need to elicit cross-reactive neutralizing antibodies and a strong cell-mediated immune response against multiple HIV-1 antigens to confer protection against challenge. In this study, DNA vaccines were constructed to express virally regulated human immunodeficiency virus-like particles (VLP) to elicit broad-spectrum immune responses to multiple HIV-1 antigens. VLPs were efficiently produced using sequences encoding *gag* and *pol* gene products from an X4 isolate and sequences encoding for *tat*, *rev*, *vpu*, and *env* from R5 or R5X4 isolates. The *integrase*, *vpr*, *vif*, and *nef* genes were deleted. In addition, the long terminal repeats (LTRs) were removed and transcription of the VLP insert was driven by the addition of the cytomegalovirus immediate-early (CMV-IE) promoter. A second generation of VLP vaccine plasmids was constructed with mutations engineered into the VLP DNA to produce particles deficient in activities associated with viral reverse transcriptase and protease. Primate cell lines, transiently transfected with DNA, efficiently secreted VLP into the supernatant that banded within a sucrose gradient at densities similar to infectious virions. In addition, these particles incorporated Env on the particle surface that bound soluble human CD4. These VLPs provide a safe and efficient strategy for presenting multiple HIV-1 antigens, expressed from a single insert, to the immune system in a structure that mimics the infectious virion.

Keywords: Human immunodeficiency virus type-1; VLP; DNA vaccine

Introduction

Approximately 42 million people are infected with the human immunodeficiency virus type 1 (HIV-1) (UNAIDS, www. unaids.org). The virus continues to spread to new populations adding to the total number of infected individuals. The use of highly active anti-retroviral therapy (HAART) has enhanced both the longevity and quality of life for infected individuals by controlling viral replication (Hogg et al., 1999). Despite the effectiveness of HAART, several drawbacks are accompanied with this treatment that limit its worldwide use. First, HAART neither protects patients against initial infection, nor does HAART clear viral infection. Second, treatment is expensive particularly for individuals living in developing nations. Third, there can be severe side effects and patients on HAART have difficulties adhering to the drug regimens. Therefore, several preventive measures to combat the spread of HIV infection have focused on the development of safe, inexpensive, and efficacious vaccines.

Many of the successful viral vaccines administered to patients, such as vaccines developed for the measles or mumps viruses, consist of replication-competent virus that has been attenuated to prevent disease (Dorner and Barrett, 1999; Jones, 2002). The experimental use of live-attenuated simian immunodeficiency virus (SIV) elicits protective immunity in nonhuman primates challenged with SIV

* Corresponding author. Division of Infectious Diseases, Department of Medicine, School of Medicine, University of Pittsburgh, Scaife Hall, Room S871, 3550 Terrace Street, Pittsburgh, PA 15261. Fax: +1 412 648 8455.
E-mail address: tmr15@pitt.edu (T.M. Ross).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.07.009

or SHIV (a hybrid virus containing the *env*, *tat*, *vpu*, and *rev* gene sequences from HIV in a SIV *gag–pol* backbone) infection (Kumar et al., 2000; Nilsson et al., 1998; Wyand et al., 1999). In addition, these monkeys had specific cell-mediated and humoral immunity that appeared to correlate with protection. However, reversion of these vaccine strains from attenuated to pathogenic forms resulted in uncontrolled replication of the virus, pathogenesis, and subsequently death of the vaccinated animals. These results observed in nonhuman primates may have a direct bearing on the use of live-attenuated HIV for vaccination against infection in humans. Consequently, this approach is not considered a viable vaccine strategy for preventing HIV or AIDS.

The development of DNA vaccine (genetic vaccine) technology has opened new avenues for AIDS vaccine research. These genetic vaccines consist of eukaryotic expression plasmids that are inoculated into target cells of a vaccinated host and subsequently translated into protein (Donnelly et al., 1998). DNA plasmids inoculated in vivo, expressing a gene insert, elicit protective immunity against a variety of pathogens (Liu et al., 1998; Robinson, 1997; Robinson and Pertmer, 2000). Similar to live-attenuated virus, DNA vaccines expressing lentiviral antigens elicit both high titer antibody and cell-mediated immune responses to HIV–SIV in both rodents and nonhuman primates (Kim et al., 2001; O'Neill et al., 2002). Therefore, administration of DNA plasmid is an alternative strategy to the use of live-attenuated virus.

The induction of a broad range of immune responses appears necessary for any vaccine strategy against HIV or AIDS (Spearman, 2003). A predominately antibody-mediated immune response does not confer protection against HIV infection (Mascola, 2003). Nonhuman primates vaccinated with plasmids expressing HIV or SIV antigens had high-titer, anti-viral antibodies but were unable to control viral challenge (Muthumani et al., 2003; Warren, 2002). In preclinical human trials, volunteers vaccinated with recombinant Env_{gp120} or Env_{gp160} elicited transient, non-neutralizing anti-Env antibodies that did not result in long-term immune memory (Lindenburg et al., 2000; Pitisuttithum et al., 2003). However, an exclusively cell-mediated immune response against HIV–SIV antigens also does not appear to correlate with long-term protective immunity (Amara et al., 2002a). Vaccine strategies that elicit both humoral and cell-mediated immunity appear to be crucial in limiting viral replication and protection from live virus infection. Monkeys primed with DNA expressing Gag–Pol and Env, followed by a vector boost of modified vaccinia Ankara (MVA) expressing the same antigens, were able to control viral replication more effectively than those animals vaccinated with only Gag–Pol (Amara et al., 2002a). These results highlight the importance of immune responses to Env, as well as to Gag–Pol, in controlling immunodeficiency virus challenges. In the present study, a DNA vaccine was constructed to express a virally regulated human immunodeficiency virus-like particle (VLP) to elicit broad-spectrum immune responses to multiple HIV-1 antigens. These VLPs were engineered with specific safety mutations and deletions in the genome to prevent integration, severely restrict RNA genome packaging, as well as inhibit reverse transcriptase and protease activity.

Results

Construction of plasmids expressing human immunodeficiency virus-like particles

In this study, a virus-like particle (VLP) was developed to elicit broad spectrum immune responses to a variety of HIV-1 antigens. The proviral plasmid pHIV-1_{BH-10}, which encodes for a CXCR4-utilizing virus, was used as a template to construct plasmids expressing VLP. A deletion was introduced into pHIV-1_{BH-10} by cloning of two PCR products encompassing (1) the regions encoding for *gag–pol* and (2) the *vpu*, *env*, *rev*, and *tat* genes (Fig. 1), which resulted in the deletion of sequences encoding *integrase*, *vif*, *vpr*, and *nef*. In addition, both long terminal repeats (LTRs) were removed. The final plasmid $pVLP_{BH10}$ expresses the capsid, matrix, nucleocapsid, protease, reverse transcriptase, envelope, Vpu, Tat, and Rev from HIV-1_{BH10}.

The $pVLP_{89.6}$ plasmid was constructed by first making two subclones encoding for the 5′ end of the VLP gene insert (*gag–pol* region) and a second subclone encoding for the 3′ end of the VLP gene insert (*vpu*, *env*, *tat*, and *rev*). Oligonucleotides were used to amplify each fragment that was then subcloned into the expression vector pTR600. The two fragments were cloned together into a single VLP gene insert (Fig. 1). Moreover, two additional 3′ PCR products were constructed using sequences from clade B viruses. Each 3′ PCR product, encoding the *env* sequences from two R5 HIV-1 isolates ADA and R2 (a kind gift from Gerald Quinnan), was cloned with the 5′ PCR product encoding for the *gag–pol* sequences from HIV-1_{BH10}. Lastly, safety mutations were introduced in the VLP DNA using site-directed mutagenesis to increase vaccine safety for potential use in humans. These mutations were designed to disable the encapsidation of viral RNA (C15S, C36S) (Lowe et al., 1991), abrogate RNase H activity (D185N, W266T, E478Q) (Chao et al., 1995; Le Grice et al., 1991; Mizrahi et al., 1994; Yamamoto et al., 1996), and inactivate the viral protease (D25N) (Babe et al., 1995; Kohl et al., 1988).

The phGag and phGag–Pol (generously donated by Gary Nabel) express core structural and enzymatic proteins from a codon-optimized gene insert (Huang et al., 2001). Each of these plasmids encodes for the Gag and Pol gene products using an X4 isolate as the pVLP plasmids (Fig.

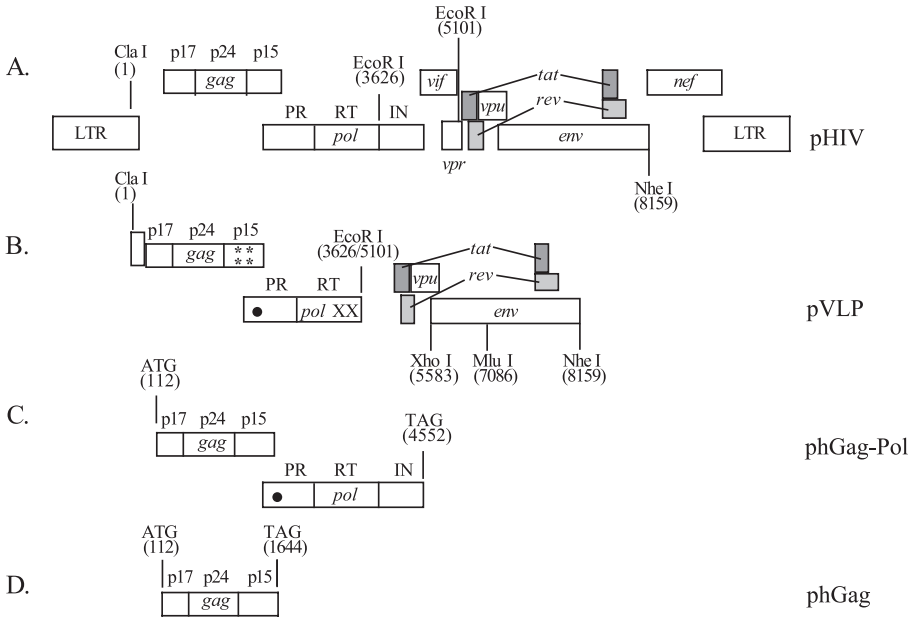

Fig. 1. Schematic representation of DNA vaccine constructs. Panel A: wild-type, proviral DNA; Panel B: VLP DNA with regions encoding for the 5′ PCR product (HIV-1_{BH10}: p17, p24, p15, PR, RT) and the 3′ PCR product (HIV-$1_{ADA,\ 89.6,\ or\ R2}$: *vpu*, *env*, *rev*, *tat*); Panel C: Gag–Pol DNA; Panel D: Gag DNA. Restriction sites are based on the nucleotide sequence for $HIV_{BH\text{-}10}$. * Represents mutations in NC. • Represents mutations in PR. X Represents mutations in RT.

1). Both plasmids were used to compare and contrast to the pVLP and $pVLP_{(D25N)}$ plasmids.

In vitro expression of human immunodeficiency virus-like particles

Each VLP expressing plasmid was verified for expression in monkey COS (Fig. 2) or human 293T cell lines (data not shown). Vaccine plasmids expressing VLP with RT and NC safety mutations, but without the protease mutation, expressed Gag and Pol gene products that were detected in both the cell lysates and supernatants of transiently transfected cells (Figs. 2A and B). Unprocessed Gag–Pol gene products (Gag–Pol_{p160}, Gag_{p55}, Gag_{p41}) were detected primarily in the cell lysate fraction compared to the supernatant (Figs. 2A and B), whereas Gag_{p24} was detected in both the cell lysates and supernatants. Similar results were observed from cells transfected with phGag–Pol (Figs. 2E and F). DNA expressing VLP with no safety mutations had similar expression patterns (data not shown). In contrast, DNA expressing VLP_{D25N} expressed incompletely processed Gag–Pol gene products (Gag_{p160}, Gag_{p55}, Gag_{p41}) (Figs. 2C and D). Fully processed Gag_{p24} or Gag_{p17} gene products were not detected in supernatants or cell lysates from cells transiently transfected with DNA expressing VLP with the PR_{D25N} (VLP_{D25N}) mutation. These results were similar to cells transfected with phGag or phGag–Pol_{D25N} (Figs. 2E and F). Env was detected in the supernatant of cells (1–2 ng/ml) transfected with DNA expressing VLP and was not affected by the introduction of safety mutations. Therefore, each of the DNA plasmids expressing virus-like particles efficiently expressed all the structural VLP gene products.

Particles were purified from the supernatants of transiently transfected cells by ultracentrifugation (20–60% sucrose gradient) and collected fractions were analyzed for particle composition and stability. Supernatants from cells transfected with DNA expressing VLP contained particles that banded between 32% and 40% sucrose (1.14–1.18 g/cm^3) (Fig. 3). These virus-like particles banded in a sucrose gradient similar to wild-type virions (Dettenhofer and Yu, 1999; Mouland et al., 2000; Tang et al., 2003; Wang et al., 1998). Gag_{p55}, Gag_{p41}, and Gag_{p24} were readily detectable in each of these fractions. All VLPs banded in the same percentage of sucrose regardless if they were expressed from DNA encoding VLP with or without the safety mutations (Fig. 3). However, supernatants from cells transfected with DNA expressing VLP with the PR_{D25N} mutation had particles without detectable levels of Gag_{p24} (Fig. 3B). Similar results were observed from cells transfected with phGag–Pol incorporating the PR_{D25N} (data not shown). However, cells transfected with the phGag–Pol plasmid produced particles that contained predominately processed Gag gene products with little higher molecular weight Gag–Pol proteins, whereas cells transfected with phGag–DNA had unprocessed gene products (Figs. 3C and D). Although each DNA plasmid efficiently expressed VLP, the composition of the particles differed depending on protease activity.

Accessory protein activity

Rev activity was not measured directly, but was observed as a result of structural HIV-1 antigen expression and

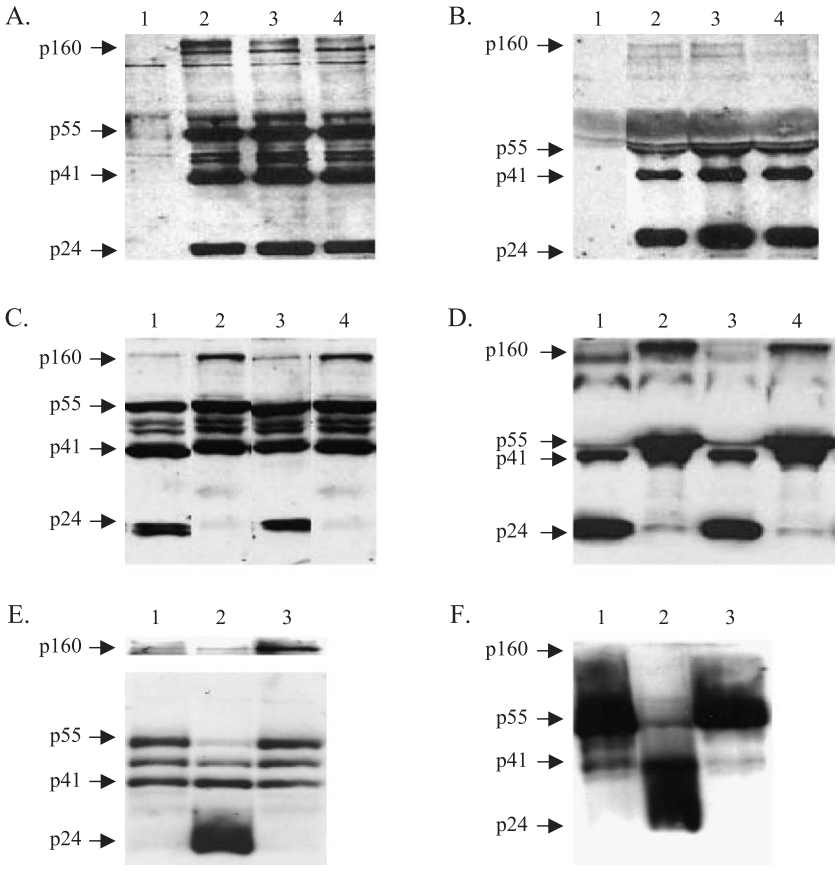

Fig. 2. Expression of vaccine constructs in vitro. COS cells were transfected with 2 μg of each vaccine plasmid. Samples were electrophoresis on a 10% Western blot (2.0% of cell lysate and 1.0% of supernatant). Panels A and B VLP with NC and RT safety mutations; lane 1, pTR600 vector; lane 2, $pVLP_{89.6}$; lane 3, $pVLP_{ADA}$; and lane 4, $pVLP_{R2}$. Panels C and D: VLP with NC and RT safety mutations and protease mutation; lane 1, $pVLP_{ADA}$; lane 2, $pVLP_{ADA(D25N)}$; lane 3, $pHIV\text{-}1_{R2}$; and lane 4, $pHIV\text{-}1_{R2(D25N)}$. Panels E and F: phGag or phGag–Pol VLP; lane 1, $phGag_{p55}$; lane 2, $phGag\text{–}Pol_{p160}$; and lane 3, $phGag\text{–}Pol_{(D25N)}$. Panels A, C, and E: cell lysates. Panels B, D, and F: supernatants.

particle formation. VLPs secreted into the supernatant of transfected cells indicate that Rev actively promoted the cytoplasmic accumulation of mRNA transcripts encoding for structural gene products (Cullen, 2003). In addition, DNA expressing infectious virions or VLP had similar levels of Tat activity as observed directly using a secreted alkaline phosphatase (SEAP) reporter assay (Berger et al., 1988). COS cells were transfected with DNA expressing infectious virions or VLP, as well as a reporter plasmid containing HIV-1 LTR-SEAP. The level of Tat activation of the LTR was similar regardless whether Tat was expressed from proviral DNA ($pHIV_{ADA}$) or DNA encoding VLP (Table 1). Therefore, DNA encoding VLP transcribed mRNA that efficiently expressed functional Tat and Rev molecules.

Envelope on the surface of VLP binds the human CD4 molecule

VLP, expressed from DNA plasmid, bound soluble human CD4 (sCD4) (Fig. 4). VLPs secreted into the supernatant of COS cells were incubated with a soluble form of human CD4. Each VLP, with $Env_{89.6}$, Env_{ADA}, or Env_{R2} on the surface, bound sCD4 with similar efficiency to wild-type, infectious HIV-1 (Fig. 4). Only VLP containing envelope, not Gag only particles, bound sCD4 indicating that the interaction was specific to particles incorporating Env. In addition, cells transfected with a plasmid expressing $gp120_{89.6}$ only did not bind to sCD4 because monomeric gp120 was not able to transverse the 20% glycerol layer during purification. Interestingly, even though the Env_{R2} on the VLP binds CCR5 independent of CD4 (unpublished observations), the Env_{R2} on the surface of the VLP bound CD4 with equal efficiency as CD4-dependent envelopes.

Discussion

In this study, a molecular clone was constructed to express a human immunodeficiency virus-like particle as a potential vaccine candidate. The composition of the expressed particle has properties that distinguish it from the many VLP for HIV-1 currently in preclinical trials (for reviews, please see Hardy et al., 2002; Spearman, 2003; Stevceva and Strober, 2004; Yao et al., 2003; Young and

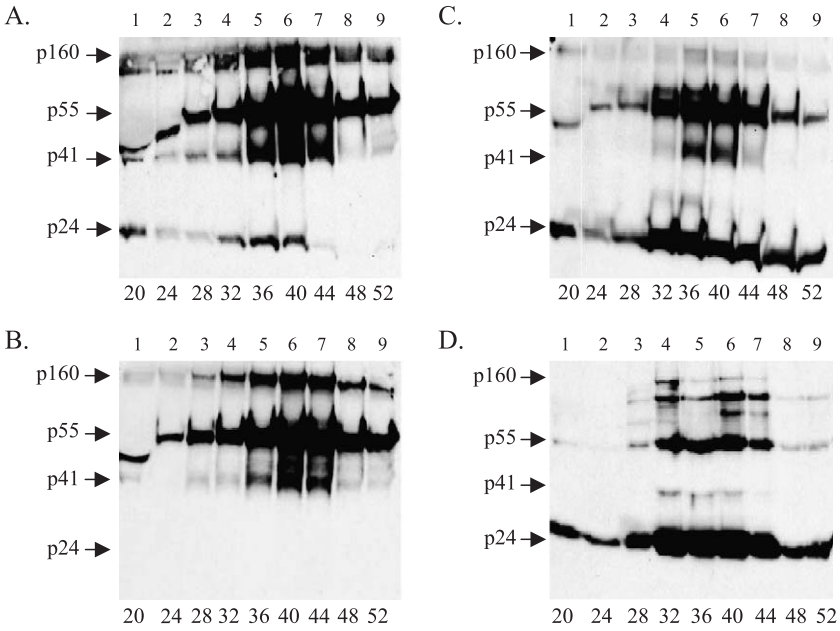

Fig. 3. Comparison of sedimentation patterns of secreted viral proteins by sucrose density-equilibrium gradient analysis. Supernatants from COS cells transiently transfected with plasmid DNA were concentrated through a 20% glycerol cushion and then subjected to 20–60% sucrose density-equilibrium gradient centrifugation. Fractions were collected (ten 1-ml aliquots) from the top of the gradient. Fractions were examined by Western analysis. Proteins were probed with HIV-Ig (1:5000) and mouse antihuman IgG (1:7000), and visualized by enhanced chemiluminescence. Panel A: $pVLP_{R2}$, panel B: $pVLP_{R2(D25N)}$, panel C: $phGag_{p55}$, panel D: $phGag–Pol_{p160}$. Percent of sucrose fraction is located at the bottom of each lane: lane 1, 20%; lane 2, 24%; lane 3, 28%; lane 4, 32%; lane 5, 36%; lane 6, 40%; lane 7, 44%; lane 8, 48%; lane 9, 52%.

Ross, 2003). In several cases, HIV, as well as SIV, proteins were expressed from more than one plasmid or the expressed particles were purified and used as an inoculum (Boyer et al., 1998; Buonaguro et al., 2002; Haigwood et al., 1999; Huang et al., 2001; Jaffy et al., 2004; Kang and Compans, 2003; Kim et al., 1997; Wang et al., 2000; Yao et al., 2002). Lentiviral particles, expressed from a single DNA plasmid or from modified vaccinia Ankara (MVA) and inoculated into nonhuman primates, elicited a broad immune response to the expressed antigens (Amara et al., 2002a, 2002b; Buge et al., 2003). The virus-like particles expressed from plasmids described in this report can be purified and used as an inoculum or the VLP can be expressed from DNA plasmid or viral vectors in vivo. The genome of the VLP contains deletions encompassing the *integrase*, *vif*, *vpr*, and *nef* genes, as well as the 5′ and 3′ long terminal repeats of HIV-1 (Fig. 1). The VLP was encoded by the HIV-1 *gag*, *pol*, *vpu*, *env*, *rev*, and *tat* gene sequences. The deletion of integrase and each viral LTR inhibits the ability of any potential VLP or proviral DNA from inserting into host cell chromosomes (Fletcher et al., 1997). The accessory genes (*vpr*, *vif*, and *nef*) have immunomodulatory effects on the immune system (Anderson and Hope, 2003), which may be deleterious for HIV vaccine development and were therefore removed. VLP DNA efficiently expressed the structural gene products (Fig. 2) and secreted particles into the supernatant of transiently transfected cells (Fig. 3). The regulatory proteins (Tat and Rev) were efficiently expressed and had similar levels of activity as proteins expressed from proviral DNA (Table 1).

Table 1
Induction of Tat activity

Plasmid	Relative percentage of Tat activity[a]
pLTR-SEAP	7.0 ± 1.3
pGag–Pol	7.5 ± 0.9
$pVLP_{ADA}$	100.0 ± 0.0
$pVLP_{89.6}$	100.0 ± 0.0
$pVLP_{R2}$	87.0 ± 8.5
$pHIV\text{-}1_{89.6}$	100.0 ± 0.0

[a] Percent of Tat activity relative to $pHIV\text{-}1_{89.6}$.

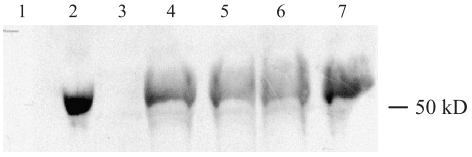

Fig. 4. VLP binding to soluble human CD4 (hCD4). Culture supernatants from COS cells transfected with plasmid DNA were incubated with supernatants from cells expressing sCD4 for 4 h and then pelleted through a 20% sucrose cushion followed by Western hybridization analysis. Proteins were probed with rabbit polyclonal anti-hCD4 (1:3000). Antibody bound protein was detected by goat anti-rabbit IgG (1:5000) and visualized by enhanced chemiluminescence. Lane 1, pTR600 vector; lane 2, purified sCD4 control; lane 3, phGag–Pol; lane 4, $pVLP_{89.6}$; lane 5, $pVLP_{R2}$; lane 6, $pVLP_{ADA}$; and lane 7, $pHIV\text{-}1_{89.6}$.

A second set of VLP expressing plasmids was constructed that contained a substitution in the region encoding for *env*. DNA gene segments encoding for *env*, as well as *tat*, *rev*, and *vpu*, were cloned into the VLP DNA backbone (Fig. 1). The *env* gene segments were cloned from one R5X4 (89.6) and two R5 isolates (ADA and R2). The ADA, along with the original 89.6 *env* genes, was selected for two reasons: (1) each envelope is currently being used by several research groups for HIV vaccine development and therefore would be useful for comparison of elicited immune responses, and (2) X4 envelopes, such as the Env derived from the HIV-1_{BH10} or HIV-1_{IIIB} isolates, are more sensitive to neutralizing antibody compared to primary R5 isolates (Crawford et al., 1999; Means et al., 1997). The Env_{ADA} is a particularly difficult envelope to neutralize and therefore, vaccine strategies that elicit neutralizing antibodies using VLP expressing this envelope may elicit a broader immune response to a diverse set of HIV-1 isolates. Env_{R2}, cloned from a patient isolate (HNS2), can be neutralized by sera from patients infected with HIV-1 from clades A, B, C, D, and F, as well as circulating recombinant forms (CRF) (Quinnan et al., 1999), and was therefore selected for use as an Env immunogen in the VLP. Virions pseudotyped with the Env_{R2} can mediate CD4-independent infection. In addition, these viruses are sensitive to neutralization by a panel of monoclonal antibodies that recognize conformation-dependent epitopes in envelope (Zhang et al., 2002). Recently, Dong et al. (2003) demonstrated that the Env_{R2}, expressed from a VEE replicon, elicited high titer neutralizing antibodies. Therefore, the 89.6, ADA, or R2 envelopes, incorporated on the surface of the VLP, each has advantages for enhancing the effectiveness of the VLP immunogen.

One advantage of incorporating viral envelope glycoproteins on the surface of the VLP is the presentation of Env in a native conformation. The inoculation of monomeric Env_{gp120} elicits high titer anti-Env antibody that does not prevent viral entry into susceptible cells. Various approaches to construct soluble trimeric forms of Env that more closely mimic the native Env_{gp160} on the surface of virions have been employed. Soluble trimerized Env_{gp140}, unstabilized or stabilized with domains (GCN4 or the T4 bacteriophage fibritin motifs or by disulfide linkers) (Abrahamyan et al., 2003; Bower et al., 2004; Sanders et al., 2000, 2002; Schulke et al., 2002; Yang et al., 2000, 2002), elicited modest levels of enhancement of neutralizing antibody compared to antibody elicited by monomeric forms of Env. However, even though many of these approaches use a trimerized envelope, there are still distinct differences between soluble, trimerized Env and the structure of native trimeric form of Env anchored on the surface of the virion. Therefore, the expression of Env in the context of a particle may prove advantageous for vaccine development.

The potential of the VLP RNA to recombine with an HIV-1 genome in an infected patient could limit the use of this vaccine in humans. Therefore, a third generation of VLP expressing plasmids was constructed that incorporated a variety of mutations to increase the safety of these vaccines for potential use in humans. The VLP was modified to prevent (1) the packaging of RNA, (2) reverse transcriptase activity, and (3) the proteolytic processing of Gag–Pol polypeptides. The first set of mutations, introduced into the nucleocapsid (NC) coding region, disrupts the two zinc finger motifs (Mizuno et al., 1996). The principal function of the NC involves the specific encapsidation of full-length, unspliced (genomic) RNA into virions. Introduction of serine residues at positions 15 and 36 in the NC prevents the binding of zinc and disrupted zinc finger formation in the NC. Previous studies have demonstrated severely restricted packaging of genome RNA into both infectious virus as well as VLP incorporating these NC mutations (Akahata et al., 2000, 2003a, 2003b; Ellenberger et al., 2004; Mizuno et al., 1996; Yovandich et al., 2001).

During the process of reverse transcription, the viral RNA is converted into proviral DNA in a series of defined steps (Acheampong et al., 2003; Freed, 2001). RT activity could have been eliminated by the deletion of the gene sequences encoding for RT. However, at least seven epitopes in RT are immunogenic and are advantageous for eliciting anti-viral immunity (Huang et al., 1995). The introduction of recombinant HIV-1 RT elicited high-titer CTL responses (Menendez-Arias et al., 1998). Therefore, we choose to disrupt RT activity without preventing the expression of this protein (Ellenberger et al., 2004) and thus the presentation of RT epitopes to the immune system.

The final mutation substituted an asparagine for aspartic acid residue at position 25 in protease. Previous studies have shown that cells transfected with DNA expressing lentiviral VLP with a mutation at amino acid 25 in PR expressed a precursor molecule with high levels of unprocessed Gag proteins (Ellenberger et al., 2004; Park and Morrow, 1991; Smith et al., 1993). Nonetheless, this precursor molecule can be incorporated into a particle through its interactions with the capsid domain of Gag (Huang et al., 1995). The viral protease, similar to other aspartyl proteases, such as renin and pepsin, contains the conserved asparagine–threonine–glycine sequence (protease amino acid positions 25–27) in the active site. The initial critical step for autoprocessing of the Gag–Pol precursor protein is the folding and dimerization of this protein leading to the formation of the active site. A substitution of an asparagine residue at this position prevents this folding and therefore results in loss of enzymatic activity without an effect on protein structure, which allows for an accumulation of unprocessed Gag_{p55} and $Gag–Pol_{p160}$ in the cell lysate and supernatants (Fig 3B). Live virus particles that are expressed from proviral DNA and contain the PR_{D25N} are noninfectious (Babe et al., 1995). In addition, co-

expression of proviral DNA and DNA expressing the PR_{D25N} resulted in a dose-dependent decrease in particle maturation and infectivity (Babe et al., 1995). Therefore, expression of these VLP vaccines in an infected patient could have the additional benefit of reducing the infectivity of the wild-type virions.

Infection with live virus results in virions containing Gag polyproteins that are fully processed during maturation post-budding. These particles are mature and composed of a dense capsid core and contain multiple viral proteins within the particle that are associated with the genomic RNA. In addition, human immunodeficiency virus-like particles are composed of immature particles with mostly processed Gag gene products and have severely reduced levels of VLP-RNA packaging (Ellenberger et al., 2004). In contrast, VLP_{D25N} is composed of surface envelope on primarily unprocessed Gag_{p160} and Gag_{p55} (Fig. 3B). Each of these various particles may have advantages for eliciting a broad range of immune responses.

The particles described in this report have distinct advantages for vaccine development. First, the deletion of the viral LTR, *integrase*, *vpr*, *vif*, and *nef* sequences enhances the safety of the vaccine and reduces the immunomodulatory effects of the immunogen. The introduction of mutations in RT and the NC further increases the safety of this vaccine by reducing the infectivity of these particles. Most live or live-attenuated viruses contain viral RNA and are therefore replication competent with the ability to produce pathogenesis in vaccinated hosts. The lack of particle-encapsidated RNA and the inability of the vaccine insert to integrate into host chromosomes are advantages of this VLP vaccine (Engelman and Craigie, 1992; Engelman et al., 1995; Lowe et al., 1991; Mizuno et al., 1996). A second advantage is that all of the HIV-1 gene products can be expressed from a single DNA plasmid. The ability to characterize one DNA plasmid expressing the entire VLP reduces the potential regulatory and safety concerns associated with testing multiple DNA plasmids for prospective use in humans. Third, VLP_{D25N} produces particles with predominately unprocessed, higher molecular weight Gag gene products that may elicit higher levels of anti-HIV CTL responses compared to particles with fully processed Gag gene products. Recently, it was demonstrated that splenocytes from mice vaccinated with particles composed of Gag_{p55} lysed a higher number of target cells, presenting Gag peptides, compared to cells from mice vaccinated with particles composed of $Gag–Pol_{p160}$ (Huang et al., 2001). Fourth, the inactivation of the viral protease may lead to markedly reduced shedding of gp120 (Hammonds et al., 2003). Human CD4 interaction increases shedding of gp120 from viral particles and gp120 may also be lost from VLP during particle purification procedures (Hart et al., 1991; McKeating et al., 1991; Moore et al., 1993). Thus, VLP_{D25N} has the added advantage of retaining Env and thereby increasing anti-Env immune responses. Lastly, the VLPs described in this report have the added advantage of presenting Env epitopes to the immune system and therefore eliciting both a broad spectrum antibody and cell-mediated immune response.

An effective vaccine against HIV or AIDS will most likely need to elicit high levels of cross-reactive neutralizing antibodies in combination with a robust cell-mediated response against multiple viral antigens to protect from disease in an infected host. One approach to achieve these goals is the development of a human immunodeficiency virus-like particle. In general, these particle-based strategies present multiple viral proteins in a conformation similar to the live virus without the safety issues associated with replication-competent virions. Current virus-like particle vaccines are similar in nature to live-attenuated viruses, expressing both structural and enzymatic proteins. The virus-like particle vaccine described in this study does not produce a productive infection or integrate into host chromosomes. The non-replicative nature of this type of vaccine prohibits the particles from reverting or recombining with wild-type vRNA to produce to a pathogenic form. The incorporation of additional safety mutations enhances the value of these vaccines for use in humans.

Materials and methods

DNA Plasmids

The vaccine plasmid pTR600 has been previously described (Green et al., 2003). Briefly, the vector was constructed to contain the cytomegalovirus immediate-early promoter (CMV-IE) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (kan^r) for selection in antibiotic media.

The parental plasmid $pHIV\text{-}1_{BH10}$ (accession number M1564) encodes the sequences from the proviral HIV-1 isolate BH10 (X4) and was used as a template to construct the plasmid $pVLP_{BH10}$ (Fig. 1). A second plasmid $pVLP_{89.6}$ expressed a VLP with the Env region encoded from the isolate 89.6 (accession number U39362). The $pVLP_{89.6}$ plasmid encodes for the following gene sequences: $HIV\text{-}1_{BH10}$ *gag–pol* ($pHIV_{BH10}$ nt 112–3626) and $HIV\text{-}1_{89.6}$ *vpu*, *env*, *rev*, *tat* ($pHIV_{BH10}$ nt 5101–8159).

The $pVLP_{89.6}$ was constructed from two subclones encoding for the 5′ end of the VLP gene insert and a second subclone encoding for the 3′ end of the VLP gene insert (Fig. 1). Oligonucleotides corresponding to the *gag–pol* sequences were used in a PCR reaction to amplify a fragment of DNA (5′ PCR product) composed

of the 5′ untranslated leader sequence (105 nucleotides) and *gag–pol* sequences (start codon of *gag* to the TAG stop codon at the 3′ end of the reverse transcriptase sequence, $pHIV_{BH10}$ nt 112–3626). For the 5′ PCR product, a *Cla*I restriction enzyme site was introduced ($pHIV_{BH10}$, nt 1) at the 5′ end of the vaccine insert, while an *Eco*RI ($pHIV_{BH10}$, nt 3626) and *Nhe*I site ($pHIV_{BH10}$, nt 8159) were added at the 3′ end of the 5′ PCR product by PCR. The following primers were used to amplify the 5′ PCR product: sense primer *5′-gagctctatcgatgcaggactcggcttgc-3′* and antisense primer *5′-ggcaggttttaatcgctagcctatgaattcc-3′*. The 3′ PCR product was a DNA fragment encoding the *vpu*, *env*, *rev*, and *tat* genes and the appropriate splice acceptor sites. An *Eco*RI site was introduced into the 5′ terminus of the 3′ PCR product ($pHIV_{BH10}$ nt 5101) and an *Nhe*I site was introduced at the 3′ terminus ($pHIV_{BH10}$ nt 8159) and the 3′ PCR product was amplified by the following primers: sense primer *5′-cccaccttaagacgttgttgacgacaaatacg-3′* and antisense primer *5′-ccacactactttcggaccgctagccaccc-3′*. Both of the 5′ and 3′ PCR products were cloned into pTR600 using unique restriction enzymes sites (5′ PCR product: *Cla*I and *Eco*RI and the 3′ PCR product using *Eco*RI and *Nhe*I). The resulting plasmid $pVLP_{89.6}$ encodes for a virus-like particle.

Two additional VLP expressing DNA plasmids were constructed that encoded for various *env* sequences. The *vpu*, *env*, *rev*, and *tat* sequences were cloned by PCR amplification from the proviral DNA from the HIV-1 isolates ADA or R2 and each PCR fragment was inserted into the $pVLP_{89.6}$ Gag–Pol backbone using unique *Eco*RI and *Bam*HI restriction sites (Fig. 1). The splice–acceptor sites were not mutated to allow for efficient mRNA processing of singly and multiply spliced messenger RNAs.

psCD4 contains the soluble human CD4 gene (four extracellular domains) and was cloned into pTR600 using unique restriction sites *Hin*dIII and *Nhe*I (sense primer *5′-gtcagcaagcttatgaaccggggagtcc-3′* and antisense primer *5′-gctgacgctagcgctacctcctccgcctccatcgatgctacctccgcctccgctacctccgccgcttccgaattcggtggaccatgtg-3′*). The plasmids $pGag_{p55}$ and $pGag_{p160}$ are derived from codon-optimized sequences (hGag and hGag–Pol, respectively) previously described (Huang et al., 2001). Sequences encoding for Gag_{p55} and $Gag–Pol_{p160}$ ($pHIV_{BH10}$, nt 112–1644 and 112–4552, respectively) were cloned from phGag and phGag–Pol (generously donated by Gary Nabel) into pTR600 using unique restrictions sites *Hin*dIII and *Bam*HI.

The plasmids were amplified in *Escherichia coli* strain-DH5 alpha, purified using anion-exchange resin columns (Qiagen, Valencia, CA), and stored at −20 °C in dH_2O. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260 and 280 nm.

Safety mutations

Safety mutations were introduced into the VLP vaccine inserts or phGag–Pol by PCR-based mutagenesis (Stratagene, La Jolla, CA) (Table 2). All mutations were verified by sequencing.

Table 2
Oligonucleotide pairs used for mutagenesis

Nucleocapsid (NC)
C15S ($pHIV_{BH10}$, nt 1285) (Mizuno et al., 1996)
sense primer *5′ggttaagagcttcaatagcggcaaagaagggc3′*
antisense primer *5′gcccttctttgccgctattgaagctcttaacc3′*
C36S ($pHIV_{BH10}$, nt 1357) (Lowe et al., 1991)
sense primer *5′gggcagctggaaaagcggaaaggaagg3′*
antisense primer *5′ccttcctttccgcttttccagctgccc3′*
Polymerase (Pol)
D185N ($pHIV_{BH10}$, nt 2460) (Chao et al., 1995)
sense primer *5′-ccagacatagttatctatcaatacatgaacgattgtatgtagg-3′*
antisense primer *5′-cctacatacaaatcgttcatgtattgatagataactatgtctgg-3′*
W266T ($pHIV_{BH10}$, nt 2703) (Le Grice et al., 1991)
sense primer *5′-ggggaaattgaataccgcaagtcagatttaccc-3′*
antisense primer *5′-gggtaaatctgacttgcggtattcaatttcccc-3′*
E478Q ($pHIV_{BH10}$, nt 3339) (Yamamoto et al., 1996)
sense primer *5′-ccctaactaacacaacaaatcagaaaactcagttacagc-3′*
antisense primer *5′-gcttgtaactgagttttctgatttgttgtgttagttaggg-3′*
Protease (PR)
D25N ($pHIV_{BH10}$, nt 1683) (Babe et al., 1995)
sense primer *5′-ggaagctctattaaatacaggagcagatg-3′*
antisense primer *5′-catctgctcctgtatttaatagagcttcc-3′*

Underlined codons represent mutations introduced into each gene sequence.

Transfections and expression analysis

The monkey fibroblast cell line COS (5×10^5 cells/transfection) was transfected with 2 μg of DNA using 12% lipofectamine according to the manufacturer's guidelines (Life Technologies, Grand Island, NY). Supernatants (2 ml) were collected and stored at −20 °C. Cell lysates were collected in 500 μl of 1% Triton X-100 and stored at −20 °C. Quantitative antigen capture ELISAs were conducted according to the manufacturer's protocol (Perkin-Elmer Life Sciences, Boston, MA).

For Western hybridization analysis, 3.3% of supernatant and 1.5% of the cell lysate were diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA), boiled for 5 min, and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and incubated with a 1:5000 dilution of polyclonal human HIV-infected patient antisera (HIV-Ig) in PBS containing 0.05% Tween 20 and 5% nonfat dry milk. After extensive washing, bound human antibodies were detected using a 1:7000 dilution of horseradish peroxidase-conjugated goat antihuman antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

For determination of Tat activity, COS cells (5×10^5) were transiently transfected using 12% lipofectamine in DMEM

with 1 μg of each VLP DNA and 1 μg of the reporter plasmid pLTR-SEAP (Berger et al., 1988). After 18 h, cells were replenished with fresh media (1.5 ml) and incubated an additional 48 h. Collected supernatants (100 μl) were incubated with 100 μl of SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM L-homoarginine) for 10 min at 37 °C followed by the addition of 20 μl *p*-nitrophenyl phosphate (120 mM) (PNP) (Sigma, St. Louis, MO) for 30 min at 37 °C. Samples were analyzed for color change at 405 nm. Data were recorded as the average of the three independent experiments ± standard deviation.

Purification of virus-like particles

Supernatants from COS cells, transiently transfected with plasmid expressing Gag, Gag–Pol, VLP, or infectious virions, were pelleted via ultracentrifugation (100 000 × *g* through 20% glycerol, weight per volume) for 2 h at 4 °C. The pellets were subsequently resuspended in PBS and overlaid onto 20–60% sucrose gradients (11 steps, 4% increments) and ultracentrifuged for 17 h at 100 000 × *g* at 4 °C. Eleven fractions (20–60%, 1 ml, weight per volume) were collected top to bottom from the gradient, and the proteins were precipitated with equal volumes of 20% trichloroacetic acid (TCA) and subjected to SDS-PAGE and immunoblotting. The viral proteins were detected by HIV-Ig via Western hybridization.

VLP binding to human CD4

Supernatants from COS cells transiently transfected with plasmids expressing VLP, infectious virions, or Gag–Pol were incubated at RT for 4 h with supernatants from COS cells transiently transfected with a plasmid expressing soluble human CD4 (sCD4) (9:1 ratio). The mixture (10 ml) was centrifuged (100 000 × *g*) and pelleted through 20% sucrose. Each pellet was resuspended in PBS and analyzed by Western hybridization for CD4 bound to VLP. sCD4 was detected by mouse polyclonal anti-CD4 (1:3000) followed by goat anti-mouse IgG conjugated to HRP (1:7000) (Bio-Rad). The proteins were enhanced by chemiluminescence and visualized by autoradiography.

Acknowledgments

We would like to thank Gary Nabel for providing the plasmids expressing Gag and Gag–Pol gene sequences and Gerald Quinnan for providing the plasmid expressing Env_{R2}. HIV-Ig, sCD4, and recombinant Env_{gp120} were obtained from the National Institute of Health AIDS Research and Reference Reagent Program. We also thank Joseph Bower for critical reading and comments. This research was supported by awards AI49061 and AI51213 from the National Institute of Allergy and Infectious Diseases to T.M.R.

References

Abrahamyan, L.G., Markosyan, R.M., Moore, J.P., Cohen, F.S., Melikyan, G.B., 2003. Human immunodeficiency virus type 1 Env with an intersubunit disulfide bond engages coreceptors but requires bond reduction after engagement to induce fusion. J. Virol. 77, 5829–5836.

Acheampong, E., Rosario-Otero, M., Dornburg, R., Pomerantz, R.J., 2003. Replication of lentiviruses. Front. Biosci. 8, s156–s174.

Akahata, W., Ido, E., Shimada, T., Katsuyama, K., Yamamoto, H., Uesaka, H., Ui, M., Kuwata, T., Takahashi, H., Hayami, M., 2000. DNA vaccination of macaques by a full genome HIV-1 plasmid which produces noninfectious virus particles. Virology 275, 116–124.

Akahata, W., Ido, E., Akiyama, H., Uesaka, H., Enose, Y., Horiuchi, R., Kuwata, T., Goto, T., Takahashi, H., Hayami, M., 2003a. DNA vaccination of macaques by a full-genome simian/human immunodeficiency virus type 1 plasmid chimera that produces non-infectious virus particles. J. Gen. Virol. 84, 2237–2244.

Akahata, W., Ido, E., Hayami, M., 2003b. Mutational analysis of two zinc-finger motifs in the nucleocapsid protein of simian immunodeficiency virus mac239. J. Gen. Virol. 84, 1641–1648.

Amara, R.R., Smith, J.M., Staprans, S.I., Montefiori, D.C., Villinger, F., Altman, J.D., O'Neil, S.P., Kozyr, N.L., Xu, Y., Wyatt, L.S., Earl, P.L., Herndon, J.G., McNicholl, J.M., McClure, H.M., Moss, B., Robinson, H.L., 2002a. Critical role for Env as well as Gag–Pol in control of a simian-human immunodeficiency virus 89.6P challenge by a DNA prime/recombinant modified vaccinia virus Ankara vaccine. J. Virol. 76, 6138–6146.

Amara, R.R., Villinger, F., Staprans, S.I., Altman, J.D., Montefiori, D.C., Kozyr, N.L., Xu, Y., Wyatt, L.S., Earl, P.L., Herndon, J.G., McClure, H.M., Moss, B., Robinson, H.L., 2002b. Different patterns of immune responses but similar control of a simian-human immunodeficiency virus 89.6P mucosal challenge by modified vaccinia virus Ankara (MVA) and DNA/MVA vaccines. J. Virol. 76, 7625–7631.

Anderson, J.L., Hope, T.J., 2003. Recent Insights into HIV accessory proteins. Curr. Infect. Dis. Rep. 5, 439–450.

Babe, L.M., Rose, J., Craik, C.S., 1995. Trans-dominant inhibitory human immunodeficiency virus type 1 protease monomers prevent protease activation and virion maturation. Proc. Natl. Acad. Sci. U.S.A. 92, 10069–10073.

Berger, J., Hauber, J., Hauber, R., Geiger, R., Cullen, B., 1988. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene 66, 1–10.

Bower, J.F., Yang, X., Sodroski, J., Ross, T.M., 2004. Elicitation of improved neutralizing antibodies using DNA vaccines expressing soluble stabilized HIV-1 envelope glycoprotein trimers conjugated to C3d. J. Virol. 78, 4710–4719.

Boyer, J., Chattergoon, M., Shah, A., Ginsberg, R., MacGregor, R., Weiner, D., 1998. HIV-1 DNA based vaccine induces a CD8 mediated cross-clade CTL response. Dev. Biol. Stand. 95, 147–153.

Buge, S.L., Ma, H.L., Amara, R.R., Wyatt, L.S., Earl, P.L., Villinger, F., Montefiori, D.C., Staprans, S.I., Xu, Y., Carter, E., O'Neil, S.P., Herndon, J.G., Hill, E., Moss, B., Robinson, H.L., McNicholl, J.M., 2003. Gp120-alum boosting of a Gag–Pol–Env DNA/MVA AIDS vaccine: poorer control of a pathogenic viral challenge. AIDS Res. Hum. Retroviruses 19, 891–900.

Buonaguro, L., Racioppi, L., Tornesello, M.L., Arra, C., Visciano, M.L., Biryahwaho, B., Sempala, S.D., Giraldo, G., Buonaguro, F.M., 2002. Induction of neutralizing antibodies and cytotoxic T lymphocytes in Balb/c mice immunized with virus-like particles presenting a gp120 molecule from a HIV-1 isolate of clade A. Antiviral Res. 54, 189–201.

Chao, S.F., Chan, V.L., Juranka, P., Kaplan, A.H., Swanstrom, R., Hutchison III, C.A., 1995. Mutational sensitivity patterns define critical residues in the palm subdomain of the reverse transcriptase of human immunodeficiency virus type 1. Nucleic Acids Res. 23, 803–810.

Crawford, J.M., Earl, P.L., Moss, B., Reimann, K.A., Wyand, M.S., Manson, K.H., Bilska, M., Zhou, J.T., Pauza, C.D., Parren, P.W.,

Burton, D.R., Sodroski, J.G., Letvin, N.L., Montefiori, D.C., 1999. Characterization of primary isolate-like variants of simian-human immunodeficiency virus. J. Virol. 73, 10199–10207.

Cullen, B.R., 2003. Nuclear mRNA export: insights from virology. Trends Biochem. Sci. 28, 419–424.

Dettenhofer, M., Yu, X.F., 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. J. Virol. 73, 1460–1467.

Dong, M., Zhang, P.F., Grieder, F., Lee, J., Krishnamurthy, G., VanCott, T., Broder, C., Polonis, V.R., Yu, X.F., Shao, Y., Faix, D., Valente, P., Quinnan Jr., G.V., 2003. Induction of primary virus-cross-reactive human immunodeficiency virus type 1-neutralizing antibodies in small animals by using an alphavirus-derived in vivo expression system. J. Virol. 77, 3119–3130.

Donnelly, J.J., Ulmer, J.B., Liu, M.A., 1998. DNA vaccines. Dev. Biol. Stand. 95, 43–53.

Dorner, F., Barrett, P.N., 1999. Vaccine technology: looking to the future. Ann. Med. 31, 51–60.

Ellenberger, D., Li, B., Smith, J., Yi, H., Folks, T., Robinson, H., Butera, S., 2004. Optimization of a multi-gene HIV-1 recombinant subtype CRF02_AG DNA vaccine for expression of multiple immunogenic forms. Virology 319, 118–130.

Engelman, A., Craigie, R., 1992. Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function in vitro. J. Virol. 66, 6361–6369.

Engelman, A., Englund, G., Orenstein, J.M., Martin, M.A., Craigie, R., 1995. Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. J. Virol. 69, 2729–2736.

Fletcher III, T.M., Soares, M.A., McPhearson, S., Hui, H., Wiskerchen, M., Muesing, M.A., Shaw, G.M., Leavitt, A.D., Boeke, J.D., Hahn, B.H., 1997. Complementation of integrase function in HIV-1 virions. EMBO J. 16, 5123–5138.

Freed, E.O., 2001. HIV-1 replication. Somatic Cell Mol. Genet. 26, 13–33.

Green, T.D., Montefiori, D.C., Ross, T.M., 2003. Enhancement of antibodies to the human immunodeficiency virus type 1 envelope by using the molecular adjuvant C3d. J. Virol. 77, 2046–2055.

Haigwood, N., Pierce, C., Robertson, M., Watson, A., Montefiori, D., Rabin, M., Lynch, J., Kuller, L., Thompson, J., Morton, W., Benvenister, R., Hu, S., Greenberg, P., Mossman, S., 1999. Protection from pathogenic SIV challenge using multigenic DNA vaccines. Immunol. Lett. 66, 183–188.

Hammonds, J., Chen, X., Ding, L., Fouts, T., De Vico, A., zur Megede, J., Barnett, S., Spearman, P., 2003. Gp120 stability on HIV-1 virions and Gag–Env pseudovirions is enhanced by an uncleaved Gag core. Virology 314, 636–649.

Hardy, G.A., Imami, N., Gotch, F.M., 2002. Improving HIV-specific immune responses in HIV-infected patients. J. HIV Ther. 7, 40–45.

Hart, T.K., Kirsh, R., Ellens, H., Sweet, R.W., Lambert, D.M., Petteway, J.S.R., Leary, J., Bugelski, P.J., 1991. Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. Proc. Natl. Acad. Sci. U.S.A. 88, 2189–2193.

Hogg, R.S., Yip, B., Kully, C., Craib, K.J., O'Shaughnessy, M.V., Schechter, M.T., Montaner, J.S., 1999. Improved survival among HIV-infected patients after initiation of triple-drug antiretroviral regimens. CMAJ 160, 659–665.

Huang, M., Orenstein, J.M., Martin, M.A., Freed, E.O., 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J. Virol. 69, 6810–6818.

Huang, Y., Kong, W.P., Nabel, G.J., 2001. Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. J. Virol. 75, 4947–4951.

Jaffy, A., Shepard, E., van Harmelen, J., Williamson, C., Williamson, A.L., Rybicki, E.P., 2004. Human immunodeficiency virus type 1 subtype C Gag virus-like particle boost substantially improves the immune response to a subtype C gag DNA vaccine in mice. J. Gen. Virol. 85, 409–413.

Jones, T., 2002. Varivax (Merck and Co). Curr. Opin. Invest. Drugs 3, 54–57.

Kang, S.M., Compans, R.W., 2003. Enhancement of mucosal immunization with virus-like particles of simian immunodeficiency virus. J. Virol. 77, 3615–3623.

Kim, J., Ayyavoo, V., Bagarazzi, M., Chattergoon, M., Boyer, J., Wang, B., Weiner, D., 1997. Development of a multicomponent candidate vaccine for HIV-1. Vaccine 15, 879–883.

Kim, J.J., Yang, J.S., Manson, K.H., Weiner, D.B., 2001. Modulation of antigen-specific cellular immune responses to DNA vaccination in rhesus macaques through the use of IL-2, IFN-gamma, or IL-4 gene adjuvants. Vaccine 19, 2496–2505.

Kohl, N.E., Emini, E.A., Schleif, W.A., Davis, L.J., Heimbach, J.C., Dixon, R.A., Scolnick, E.M., Sigal, I.S., 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. U.S.A. 85, 4686–4690.

Kumar, A., Lifson, J.D., Silverstein, P.S., Jia, F., Sheffer, D., Li, Z., Narayan, O., 2000. Evaluation of immune responses induced by HIV-1 gp120 in rhesus macaques: effect of vaccination on challenge with pathogenic strains of homologous and heterologous simian human immunodeficiency viruses. Virology 274, 149–164.

Le Grice, S.F., Naas, T., Wohlgensinger, B., Schatz, O., 1991. Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. EMBO J. 10, 3905–3911.

Lindenburg, K., Langendam, M.W., Veenstra, J., Miedema, F., Coutinho, R., 2000. Long-term effects of therapeutic vaccination with rgp 120. AIDS 14, 203–204.

Liu, M.A., Fu, T.M., Donnelly, J.J., Caulfield, M.J., Ulmer, J.B., 1998. DNA vaccines. Mechanisms for generation of immune responses. Adv. Exp. Med. Biol. 452, 187–191.

Lowe, D.M., Parmar, V., Kemp, S.D., Larder, B.A., 1991. Mutational analysis of two conserved sequence motifs in HIV-1 reverse transcriptase. FEBS Lett. 282, 231–234.

Mascola, J.R., 2003. Defining the protective antibody response for HIV-1. Curr. Mol. Med. 3, 209–216.

McKeating, J.A., McKnight, A., Moore, J.P., 1991. Differential loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: effects on infectivity and neutralization. J. Virol. 65, 852–860.

Means, R.E., Greenough, T., Desrosiers, R.C., 1997. Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J. Virol. 71, 7895–7902.

Menendez-Arias, L., Mas, A., Domingo, E., 1998. Cytotoxic T-lymphocyte responses to HIV-1 reverse transcriptase. Viral Immunol. 11, 167–181.

Mizrahi, V., Brooksbank, R.L., Nkabinde, N.C., 1994. Mutagenesis of the conserved aspartic acid 443, glutamic acid 478, asparagine 494, and aspartic acid 498 residues in the ribonuclease H domain of p66/p51 human immunodeficiency virus type I reverse transcriptase. Expression and biochemical analysis. J. Biol. Chem. 269, 19245–19249.

Mizuno, A., Ido, E., Goto, T., Kuwata, T., Nakai, M., Hayami, M., 1996. Mutational analysis of two zinc finger motifs in HIV type 1 nucleocapsid proteins: effects on proteolytic processing of Gag precursors and particle formation. AIDS Res. Hum. Retroviruses 12, 793–800.

Moore, J.P., Burkly, L.C., Connor, R.I., Cao, Y., Tizard, R., Ho, D.D., Fisher, R.A., 1993. Adaptation of two primary human immunodeficiency virus type 1 isolates to growth in transformed T cell lines correlates with alterations in the responses of their envelope glycoproteins to soluble CD4. AIDS Res. Hum. Retroviruses 9, 529–536.

Mouland, A.J., Mercier, J., Luo, M., Bernier, L., DesGroseillers, L., Cohen, E.A., 2000. The double-stranded RNA-binding protein Staufen is incorporated in human immunodeficiency virus type 1: evidence for a role in genomic RNA encapsidation. J. Virol. 74, 5441–5451.

Muthumani, K., Bagarazzi, M., Conway, D., Hwang, D.S., Manson, K., Ciccarelli, R., Israel, Z., Montefiori, D.C., Ugen, K., Miller, N., Kim, J., Boyer, J., Weiner, D.B., 2003. A Gag–Pol/Env–Rev SIV239 DNA vaccine improves CD4 counts, and reduce viral loads after pathogenic intrarectal SIV(mac)251 challenge in rhesus macaques. Vaccine 21, 629–637.

Nilsson, C., Makitalo, B., Thorstensson, R., Norley, S., Binninger-Schinzel, D., Cranage, M., Rud, E., Biberfeld, G., Putkonen, P., 1998. Live attenuated simian immunodeficiency virus (SIV)mac in macaques can induce protection against mucosal infection with SIVsm. AIDS 12, 2261–2270.

O'Neill, E., Martinez, I., Villinger, F., Rivera, M., Gascot, S., Colon, C., Arana, T., Sidhu, M., Stout, R., Montefiori, D.C., Martinez, M., Ansari, A.A., Israel, Z.R., Kraiselburd, E., 2002. Protection by SIV VLP DNA prime/protein boost following mucosal SIV challenge is markedly enhanced by IL-12/GM-CSF co-administration. J. Med. Primatol. 31, 217–227.

Park, J., Morrow, C.D., 1991. Overexpression of the gag–pol precursor from human immunodeficiency virus type 1 proviral genomes results in efficient proteolytic processing in the absence of virion production. J. Virol. 65, 5111–5117.

Pitisuttithum, P., Nitayaphan, S., Thongcharoen, P., Khamboonruang, C., Kim, J., de Souza, M., Chuenchitra, T., Garner, R.P., Thapinta, D., Polonis, V., Ratto-Kim, S., Chanbancherd, P., Chiu, J., Birx, D.L., Duliege, A.M., McNeil, J.G., Brown, A.E., 2003. Safety and immunogenicity of combinations of recombinant subtype E and B human immunodeficiency virus type 1 envelope glycoprotein 120 vaccines in healthy Thai adults. J. Infect. Dis. 188, 219–227.

Quinnan Jr., G.V., Zhang, P.F., Fu, D.W., Dong, M., Alter, H.J., 1999. Expression and characterization of HIV type 1 envelope protein associated with a broadly reactive neutralizing antibody response. AIDS Res. Hum. Retroviruses 15, 561–570.

Robinson, H.L., 1997. DNA vaccines for immunodeficiency viruses. AIDS 11 (Suppl. A), S109–S119.

Robinson, H.L., Pertmer, T.M., 2000. DNA vaccines for viral infections: basic studies and applications. Adv. Virus Res. 55, 1–74.

Sanders, R.W., Schiffner, L., Master, A., Kajumo, F., Guo, Y., Dragic, T., Moore, J.P., Binley, J.M., 2000. Variable-loop-deleted variants of the human immunodeficiency virus type 1 envelope glycoprotein can be stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits. J. Virol. 74, 5091–5100.

Sanders, R.W., Vesanen, M., Schuelke, N., Master, A., Schiffner, L., Kalyanaraman, R., Paluch, M., Berkhout, B., Maddon, P.J., Olson, W.C., Lu, M., Moore, J.P., 2002. Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. J. Virol. 76, 8875–8889.

Schulke, N., Vesanen, M.S., Sanders, R.W., Zhu, P., Lu, M., Anselma, D.J., Villa, A.R., Parren, P.W., Binley, J.M., Roux, K.H., Maddon, P.J., Moore, J.P., Olson, W.C., 2002. Oligomeric and conformational properties of a proteolytically mature, disulfide-stabilized human immunodeficiency virus type 1 gp140 envelope glycoprotein. J. Virol. 76, 7760–7776.

Smith, A.J., Srinivasakumar, N., Hammarskjold, M.L., Rekosh, D., 1993. Requirements for incorporation of Pr160gag–pol from human immunodeficiency virus type 1 into virus-like particles. J. Virol. 67, 2266–2275.

Spearman, P., 2003. HIV vaccine development: lessons from the past and promise for the future. Curr. HIV Res. 1, 101–120.

Stevceva, L., Strober, W., 2004. Mucosal HIV vaccines: where are we now? Curr. HIV Res. 2, 1–10.

Tang, S., Murakami, T., Cheng, N., Steven, A.C., Freed, E.O., Levin, J.G., 2003. Human immunodeficiency virus type 1 N-terminal capsid mutants containing cores with abnormally high levels of capsid protein and virtually no reverse transcriptase. J. Virol. 77, 12592–15602.

UNAIDS, www.unaids.org.

Wang, C.T., Lai, H.Y., Li, J.J., 1998. Analysis of minimal human immunodeficiency virus type 1 gag coding sequences capable of virus-like particle assembly and release. J. Virol. 72, 7950–7959.

Wang, S., Kozlowski, P., Schmelz, G., Manson, K., Wyand, M., Glickman, R., Montefiori, D., Lifson, J., Johnson, R., Neutra, M., Aldovini, A., 2000. Effective induction of simian immunodeficiency virus-specific systemic and mucosal immune responses in primates by vaccination with proviral DNA producing intact but noninfectious virions. J. Virol. 74, 10514–10522.

Warren, J., 2002. Preclinical AIDS vaccine research: survey of SIV, SHIV, and HIV challenge studies in vaccinated nonhuman primates. J. Med. Primatol. 31, 237–256.

Wyand, M.S., Manson, K., Montefiori, D.C., Lifson, J.D., Johnson, R.P., Desrosiers, R.C., 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J. Virol. 73, 8356–8363.

Yamamoto, S., Folks, T.M., Heneine, W., 1996. Highly sensitive qualitative and quantitative detection of reverse transcriptase activity: optimization, validation, and comparative analysis with other detection systems. J. Virol. Methods 61, 135–143.

Yang, X., Florin, L., Farzan, M., Kolchinsky, P., Kwong, P.D., Sodroski, J., Wyatt, R., 2000. Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. J. Virol. 74, 4746–4754.

Yang, X., Lee, J., Mahony, E.M., Kwong, P.D., Wyatt, R., Sodroski, J., 2002. Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin. J. Virol. 76, 4634–4642.

Yao, Q., Vuong, V., Li, M., Compans, R.W., 2002. Intranasal immunization with SIV virus-like particles (VLPs) elicits systemic and mucosal immunity. Vaccine 20, 2537–2545.

Yao, Q., Bu, Z., Vzorov, A., Yang, C., Compans, R.W., 2003. Virus-like particle and DNA-based candidate AIDS vaccines. Vaccine 21, 638–643.

Young, K.R., Ross, T.M., 2003. Particle-based vaccines for HIV-1 infection. Curr. Drug Targets Infect. Disord. 3, 151–169.

Yovandich, J.L., Chertova, E.N., Kane, B.P., Gagliardi, T.D., Bess, J.W., Sowder, R.C., Henderson, L.E., Gorelick, R.J., 2001. Alteration of zinc-binding residues of simian immunodeficiency virus p8(NC) results in subtle differences in gag processing and virion maturation associated with degradative loss of mutant NC. J. Virol. 75, 115–124.

Zhang, P.F., Bouma, P., Park, E.J., Margolick, J.B., Robinson, J.E., Zolla-Pazner, S., Flora, M.N., Quinnan Jr., G.V., 2002. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J. Virol. 76, 644–655.